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Flock House virus subgenomic RNA3 is replicated and its replication correlates with transactivation of RNA2

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Abstract

The nodavirus *Flock House virus* has a bipartite genome composed of RNAs 1 and 2, which encode the catalytic component of the RNA-dependent RNA polymerase (RdRp) and the capsid protein precursor, respectively. In addition to catalyzing replication of the viral genome, the RdRp also transcribes from RNA1 a subgenomic RNA3, which is both required for and suppressed by RNA2 replication. Here, we show that in the absence of RNA1 replication, FHV RdRp replicated positive-sense RNA3 transcripts fully and copied negative-sense RNA3 transcripts into positive strands. The two nonstructural proteins encoded by RNA3 were dispensable for replication, but sequences in the 3′-terminal 58 nucleotides were required. RNA3 variants that failed to replicate also failed to transactivate RNA2. These results imply that RNA3 is naturally produced both by transcription from RNA1 and by subsequent RNA1-independent replication and that RNA3 replication may be necessary for transactivation of RNA2.

Keywords: Flock House virus; Hemireplication; Nodavirus; RNA replication; Subgenomic RNA; Transactivation

Introduction

Flock House virus (FHV) is the most extensively studied member of the *Nodaviridae*, a family of nonenveloped icosahedral viruses with bipartite positive-sense RNA genomes (Ball and Johnson, 1998; van Regenmortel et al., 2000). The FHV genome consists of two RNA segments that are coencapsidated (Krishna and Schneemann, 1999). RNA1 encodes protein A, the catalytic subunit of the viral RNA-dependent RNA polymerase (RdRp), whereas RNA2 encodes the precursor of the mature capsid proteins. The RdRp replicates both segments of the viral genome and also transcribes from RNA1 a single subgenomic RNA (sgRNA), RNA3. This 387-nucleotide (nt) sgRNA is similar to the viral genomic RNAs in that all three are capped at their 5′ ends but have neither poly(A) tails nor recognizable tRNA-like structures at their 3′ ends (Buck, 1996; Dreher, 1999; Guarino et al., 1984). However, RNA3 is not detected in virions.

RNA3 encodes two nonstructural proteins, B1 and B2 (Friesen and Rueckert, 1982; Guarino et al., 1984). Protein B1 represents the C-terminal 102 residues of protein A but has no known function. Open reading frame (ORF) B2 overlaps and is in the +1 reading frame relative to B1. The 106-residue B2 protein is important for maintenance of RNA replication (Ball, 1995) and has recently been implicated as a suppressor of RNA interference when expressed in plants and in cultured insect cells (Li et al., 2002).

Independent of its role as a message, RNA3 is required for replication of RNA2 in a process referred to as transactivation (Eckerle and Ball, 2002). Although the mechanism of transactivation has yet to be determined, we recently mapped the RNA3-dependent replication signal in RNA2 to the 3′ end (Albariño et al., 2003). Once transactivated, RNA2 suppresses RNA3 transcription (Gallagher et al., 1983; Zhong and Rueckert, 1993). The implication of this counterregulation is that RNA3 coordinates replication of the two viral genome segments (Eckerle and Ball, 2002),

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and this possibility prompted us to investigate RNA3 synthesis in more detail.

The mechanism of RNA3 synthesis from an RNA1 template has not been determined for any member of the *Nodaviridae*. Two models have been proposed for synthesis of FHV RNA3 from RNA1: internal initiation and premature termination (Zhong and Rueckert, 1993). According to the internal initiation model, the viral RdRp initiates transcription at an internal site on a (−)-sense RNA1 template to synthesize (+)-sense RNA3. The premature termination model posits that the RdRp uses (+)-sense RNA1 to begin synthesizing (−)-sense RNA1, but terminates prematurely, generating (−)-sense RNA3. This (−)-sense RNA3 is then used as a template for synthesis of (+)-sense RNA3. While not definitive, the evidence to date suggests that FHV RNA3 synthesis occurs by a premature termination mechanism (reviewed in White, 2002). Most recently, Lindenbach et al. (2002) identified a long-distance intramolecular basepairing interaction in RNA1 that is required for RNA3 synthesis. As the critical interaction appears to occur in (+)-sense RNA1 (Lindenbach and Ahlquist, personal communication), it has been proposed that the resulting secondary structure promotes early termination that results in generation of (−)-sense RNA3 (White, 2002).

By whatever mechanism RNA1 initially templates the synthesis of subgenomic RNA3, the following observations suggest that, once made, FHV RNA3 undergoes at least hemireplication (i.e., (+) to (−) or (−) to (+)) and possibly full replication. First, (−)-sense RNA3 is produced during FHV RNA replication in diverse systems such as cultured insect, mammalian, and yeast cells (Eckerle and Ball, 2002; Price et al., 2000; Zhong and Rueckert, 1993). Second, the asymmetry of (+)- versus (−)-strand accumulation observed for genomic RNAs 1 and 2 is also observed for RNA3 (Price et al., 2000). Third, covalently linked head-to-tail homodimers of RNA3 accumulate during replication of wild-type FHV RNA, as do homodimers of the two genomic RNA segments (Albariño et al., 2003, 2001). Although the role of nodaviral RNA dimers is unclear, they are probably generated when the RdRp occasionally switches templates during RNA recombination, and their presence correlates with RNA replication (Albariño et al., 2001).

Delineation of the *cis*-acting signals necessary for replication of the genomic RNAs further suggests that RNA3 might serve as a template for full replication. We recently found that when combined with a functional 5′ replication element, the terminal 108 nt at the 3′ end of RNA1 were sufficient for its replication (Albariño et al., 2003). Since this sequence is also present at the 3′ end of RNA3, we reasoned that the sgRNA likely contained a functional 3′ *cis*-acting replication element. Earlier studies found that the 5′-terminal sequence requirements for RNA2 replication could consist of as few as 3 nt (Ball, 1994; Ball and Li, 1993). If the same applies to RNA1, then because the first 3 nt of RNA1 and RNA3 are identical (5′ GUU. . .), the 5′-terminal sequence requirements for replication might also be fulfilled for RNA3. Moreover, replication of an RNA3-RNA2 heterodimer that contained RNA3 in the upstream position yielded positive-sense RNA3 (Albariño et al., 2003). In the absence of evidence for cleavage of the heterodimer, the simplest explanation is that RNA3 contained functional *cis*-acting signals at both termini. Together, these considerations imply that, after its synthesis from RNA1, RNA3 may subsequently undergo full replication independent of an RNA1 template. This question had not yet been addressed for any nodavirus.

In the work described here, we tested the possibility of RNA3 replication. For this purpose the viral RdRp was translated from a nonreplicating message, supplied with monomeric or dimeric RNA3 templates of either polarity, and RdRp-dependent RNA products were analyzed. The ability of some of the RNA3 constructs to transactivate replication of RNA2 was also investigated.

Results

Replication of FHV RNA3

To generate RNA3 in vivo we used the transcription plasmid, FHV3(0,0), as described previously (Eckerle and Ball, 2002), and shown schematically in Fig. 1A. After transcription by bacteriophage T7 DNA-dependent RNA polymerase and autocatalytic cleavage of the transcript by the *Hepatitis delta virus* (HDV) ribozyme, the 5′ and 3′ ends of the transcript have no extraneous nucleotides. FHV RdRp was supplied from plasmid pTMFA, which expresses protein A from a nonreplicable RNA (Eckerle and Ball, 2002). Because the 5′ and 3′ UTRs of RNA1 were replaced by foreign sequences in pTMFA, the protein A mRNA transcribed from this plasmid was not a functional template either for replication or for synthesis of RNA3 by the RdRp (for example, Fig. 1B, lane 8; Eckerle and Ball, 2002). To determine whether the RdRp was active on RNA3 transcripts, we transfected plasmids FHV3(0,0) and pTMFA individually or together into BSR-T7/5 cells, which constitutively express T7 RNA polymerase in the cytoplasm (Buchholz et al., 1999). Approximately 54 h posttransfection, total cellular RNA was extracted, resolved by electrophoresis in denaturing agarose-formaldehyde gels, and analyzed by Northern blot hybridization with a probe complementary to (+)-sense RNA3 (and the 3′ end of RNA1).

In the absence of the RdRp, only the uncleaved T7 transcript (UCT) from FHV3(0,0) was detected (Fig. 1B, lane 2). The cleaved T7 transcript was detected on longer exposures (data not shown), indicating that ribozyme-mediated cleavage is inefficient in this sequence context. In the presence of the RdRp, the predominant RNA product from FHV3(0,0) transcripts (Fig. 1B, lane 9) comigrated with authentic RNA3 produced during replication either of a full-length RNA1 template in which ORF A was closed,

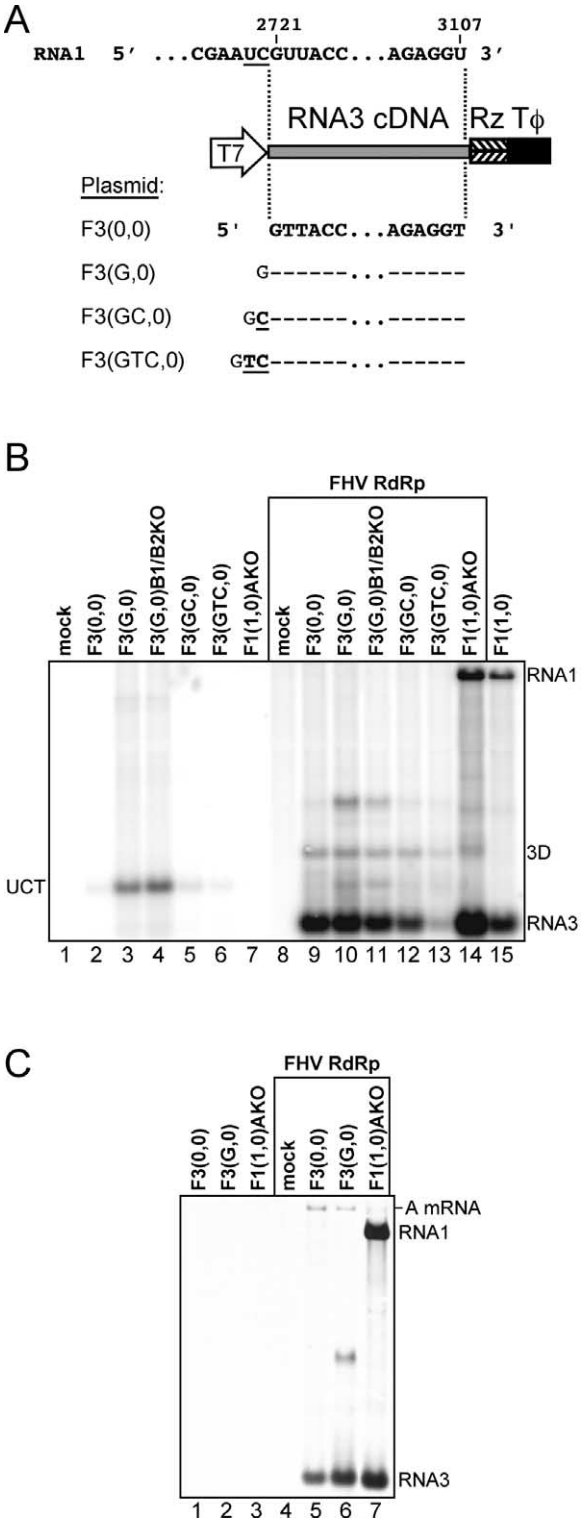

Fig. 1. Replication of FHV RNA3. (A) Schematic of FHV RNA3 transcription plasmids. Full-length cDNA of FHV RNA3 was inserted in a transcription plasmid that contained a T7 promoter (T7), cDNA of an HDV ribozyme (Rz), and a T7 transcriptional terminator (TΦ) such that transcription from the T7 promoter and autocatalytic cleavage by the ribozyme generated primary transcripts that had authentic or nearly authentic termini. FHV RNA3 and RNA1 are abbreviated as F3 and F1, respectively, in the plasmid names in this and subsequent figures. Terminal nonviral nucleotides are indicated in the plasmid names in parentheses with those at the 5′ end first, a comma, and then those at the 3′ end. Zero indicates an authentic terminal sequence. Terminal sequences of the RNA3 cDNAs are shown below the plasmid schematic; dashes indicate identity. The 3′ terminal sequence of RNA1 is shown at top for comparison. RNA1 and RNA3 sequences are shown in bold type. A nonviral G residue at the 5′ end of the RNA3 cDNAs is shown in plain type. The 2 nt immediately upstream of the RNA3 sequence within RNA1 are underlined. (B) Northen blot analysis of FHV RNA3 replication products. BSR-T7/5 cells were mock transfected (lane 1) or transfected with the indicated FHV RNA3 plasmids in the absence (lanes 2–6) or presence of an FHV protein A expression vector, pTMFA, to provide the viral RdRp (lanes 9–13). In plasmid FHV3(G,0)B1/B2KO ORFs B1 and B2 were closed. Lane 8 corresponds to cells that received pTMFA alone. For comparison, cells were transfected with a plasmid for transcription of full-length RNA1 in which ORF A was closed, FHV1(1,0)AKO, in the absence or presence of pTMFA (lanes 7 and 14). Cells were transfected with FHV1(1,0), a plasmid for transcription of autonomously replicating wild-type RNA1, as a source of authentic RNA3 (lane 15). At 54 h posttransfection total RNA was extracted, resolved in denaturing 1.5% agarose-formaldehyde gels, and transferred to nylon membranes. The blots were hybridized with an RNA probe complementary to (+)-sense RNA3. UCT indicates uncleaved primary transcripts from the RNA3 plasmids. RNAs 1 and 3 are also indicated, as are homodimers of RNA3 (3D). (C) Metabolic labeling of FHV RNA3 replication products. Cells were transfected with FHV3(0,0), FHV3(G,0), or FHV1(1,0)AKO alone (lanes 1–3) or together with pTMFA (lanes 5–7). Lane 4 corresponds to cells that received pTMFA alone. At 48 h posttransfection RNA replication products were metabolically labeled with [^{3}H]uridine in the presence of actinomycin D for 4 h. Total RNA was resolved as in Fig. 1B, and labeled RNAs were visualized by fluorography. RNAs 1 and 3 are indicated, as is a 3.7-kb species of the size expected for the T7 transcript from pTMFA (A mRNA). This latter species was also detected in the Northern presented above in Fig. 1B and in those presented below but was cropped from the figures.

FHV1(1,0)AKO, or of the autonomously replicating wild-type RNA1 replicon, FHV1(1,0) (Fig. 1B, lanes 14 and 15; Ball, 1995). This result implied that the RdRp used the FHV3(0,0) transcript as a template for production of a (−)-sense RNA3 complement, which in turn was used as a template for synthesis of progeny (+)-sense RNA3. Supporting this idea, RNA3-specific (−)-strand RNAs were detected in an RdRp-dependent manner when duplicate membranes were hybridized with a probe complementary to (−)-sense RNA3 (data not shown). Together, these results provided the first evidence that RNA3 can be fully replicated.

To further confirm that the RNA3 detected by Northern blot analysis was produced by the RdRp in a DNA-independent manner, cells were transfected with plasmids FHV3(0,0) and FHV1(1,0)AKO alone or together with pTMFA to provide the FHV RdRp, and RdRp products were specifically labeled by metabolic incorporation of [^{3}H]uridine in the presence of actinomycin D (Act D) to inhibit DNA-dependent RNA synthesis. RNAs were resolved on a denaturing gel and labeled RNAs were detected by fluorography. As was observed by Northern blot analysis, no RNA1 or RNA3 species were detected when pTMFA was transfected alone (Fig. 1C, lane 4). FHV3(0,0) transcripts were amplified in an RdRp-dependent and Act D resistant manner and comigrated with RNA3 produced during repli-

cation of an RNA1 template (Fig. 1C, compare lanes 1 and 3 with 5 and 7). This result established that the RNA3 detected by Northern blot hybridization was synthesized by the viral RdRp, and confirmed that the RdRp could fully replicate subgenomic RNA3 in the absence of a replication-competent RNA1 template.

Effects of 5′ additions on RNA3 transcripts

The levels of RNA3 transcribed from plasmid FHV3(0,0) were extremely low, presumably because of the unfavorable sequence context for initiation by T7 RNA polymerase, so we inserted an extra G residue between the T7 promoter and the RNA3 cDNA in plasmid FHV3(G,0) (Fig. 1A). Because T7 RNA polymerase initiates transcription more efficiently when the 5′ end of the transcript contains tandem G residues, the T7 transcripts from FHV3(G,0) were markedly more abundant than those from FHV3(0,0) (Fig. 1B, compare lanes 2 and 3). However, despite the increased abundance of T7 transcripts, the replication products from FHV3(G,0) were only moderately enhanced (Fig. 1B, compare lanes 9 and 10). Metabolic labeling also showed that RNA3 synthesis initiated by FHV3(G,0) was enhanced relative to FHV3(0,0) (Fig. 1C, compare lanes 5 and 6). In the case of FHV3(G,0), a minor product that migrated approximately midway down the gel was detected which was also RdRp-dependent and Act D resistant. This species was also detected by Northern blot hybridization with probes complementary to either (+)-sense RNA3 (for example, Fig. 1B, lane 10) or the T7 promoter (data not shown), indicating that this species contained non-FHV sequences derived from the vector.

When two extra G residues were included before the RNA3 cDNA, T7 transcription was further enhanced compared to FHV3(G,0), but RNA3 replication products were less abundant than with either the (0,0) or the (G,0) constructs (data not shown). This reduction suggested that the nonviral nucleotides interfered with RNA3 replication in some way, an interpretation consistent with earlier findings that terminal extensions—particularly those at the 5′ end—interfere with the template activity of FHV genomic RNAs (Ball, 1995; Ball and Li, 1993).

We reported previously that covalently linked homo- and heterodimeric RNAs are routinely produced during replication of FHV genomic RNAs and their derivatives, and we postulated that dimeric RNAs may function as intermediates in the replication of the viral genomic RNAs by serving as templates for the synthesis of progeny monomers (Albariño et al., 2001). In the current work, RNA3 dimers (3D) were produced during replication of RNA3 transcripts (for example, Fig. 1B, lane 9) and also during replication of RNA1 (Fig. 1B, lane 14) as observed previously (Albariño et al., 2001; Eckerle and Ball, 2002).

The junctions of authentic (−)-sense RNA3 dimers contain one extra G residue, which is templated by C2720, the nucleotide immediately preceding the RNA3 region of RNA1 (Albariño et al., 2001). The discovery of the extra residue in RNA3 dimer junctions suggested that (−)-sense RNA3 monomers may have an extra G at their 3′ end, which would not have a counterpart in (+)-sense RNA3, but which might be important for correct initiation of (+)-strand RNA3 synthesis. Having established that the RdRp can generate (−)-sense RNAs from (+)-sense T7 transcripts, we engineered our RNA3 transcription plasmid to have an extra C residue upstream of the RNA3 sequence in the T7 transcript. Others reported that RNA3 of the closely related *Black beetle virus* had a 5′ end corresponding to the U residue 1 nt further upstream in RNA1 (Guarino et al., 1984), so we made another construct that had 5′ UC upstream of the RNA3 sequence. To promote transcription initiation by T7 RNA polymerase, we added a 5′-terminal G before these pyrimidines, generating plasmids FHV3(GC,0) and FHV3(GTC,0) (Fig. 1A). As expected, the levels of transcripts from these plasmids were reduced relative to FHV3(G,0) (Fig. 1B, compare lanes 3, 5, and 6). More importantly, the levels of replication products resulting from FHV3(GC,0) and FHV3(GTC,0) were also reduced (Fig. 1B, compare lanes 10, 12, and 13). These results provided no support for the idea that additional nucleotides at the 3′ end of the RNA3 (−)-strand corresponding to U2719 and C2720 enhanced RNA3 replication. Therefore, FHV3(G,0) was used as the parental plasmid for all subsequent work.

RNA3 replication did not require B1 or B2 proteins

It has been previously demonstrated that both proteins encoded by RNA3 are dispensable for RNA1 replication, at least when replication was initiated from a transcription plasmid in cultured baby hamster kidney cells (Ball, 1995). To determine whether these proteins were also dispensable for RNA3 replication, we closed both ORFs (see Materials and methods for details). The pattern and level of replication products directed by the mutant plasmid FHV3(G,0)B1/B2KO were almost indistinguishable from those of the parental replicon FHV3(G,0), indicating that the B1 and B2 proteins were unnecessary for RNA3 replication (Fig. 1B, compare lanes 10 and 11). This result also showed that major changes in the translatability of RNA3 had little discernable effect on its ability to replicate.

Removal of nonviral sequences during RNA replication

To map the 5′ ends of the RNA3 replication products relative to their respective templates, primer extension (PE) analysis was performed on total cellular RNA from plasmid-transfected cells. The oligodeoxynucleotide used as primer annealed to (+)-sense RNA3 and RNA1. The predominant PE products from transcripts of FHV3(0,0) or FHV3(G,0) showed the expected 5′ ends, indicating that T7 RNA polymerase initiated transcription predominantly at the first G after the T7 promoter (Fig. 2, lanes 2 and 3). No

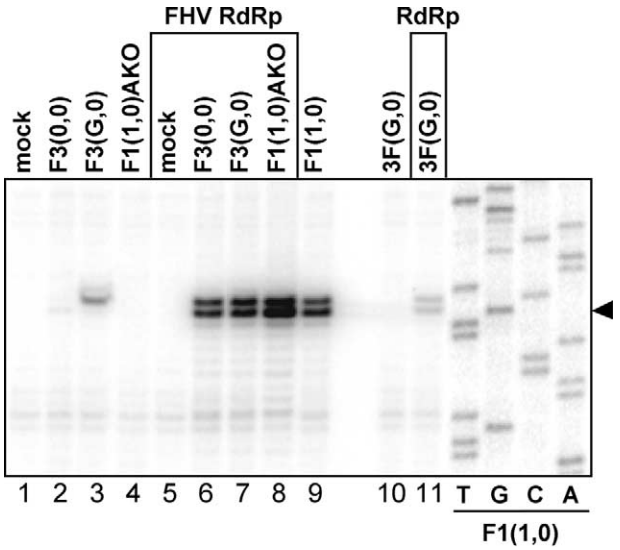

Fig. 2. Primer extension analysis of the 5′ ends of FHV RNA3 transcripts and replication products. Cells were mock transfected (lane 1) or transfected with FHV3(0,0), FHV3(G,0), or FHV1(1,0)AKO alone (lanes 2–4) or together with pTMFA (lanes 6–8). Lanes 5 and 9 correspond to cells that received only pTMFA or FHV1(1,0), respectively. Lanes 10 and 11 correspond to cells transfected with a plasmid for transcription of negative-sense RNA3, 3VHF(G,0) (abbreviated as 3F(G,0) in the figure), in the absence or presence of pTMFA, respectively. At 54 h posttransfection total RNA was extracted and an aliquot of each was subjected to primer extension (PE) analysis. The 5′ ends of T7 transcripts of RNA3 and of RNA3 replication products were mapped by annealing and extending a ^{32}P-end-labeled oligodeoxynucleotide complementary to FHV RNA3 nt 81–99. PE products were resolved in 6% polyacrylamide sequencing gels and run adjacent to a DNA sequencing ladder generated using the same oligodeoxynucleotide primer and plasmid FHV1(1,0) as template. The complement of the chain-terminating dideoxynucleotide is given below each sequencing reaction, and the first G residue of the RNA3 sequence is indicated by an arrowhead.

PE products in this size range were detected from cells that were mock transfected or transfected with FHV1(1,0)AKO or pTMFA alone (Fig. 2, lanes 1, 4, and 5).

When cells were transfected with pTMFA and either FHV3(0,0) or FHV3(G,0), PE resulted in a doublet that we attribute to capped (upper band) and uncapped (lower band) versions of RNA3 (Fig. 2, lanes 6 and 7; Davison and Moss, 1989). These doublets were indistinguishable from those resulting from replication of RNA1 transcripts (Fig. 2, lanes 8 and 9). This result confirmed that RNA3 replicated in the absence of RNA1 had 5′ ends at the authentic positions.

The cap status of the RNA templates that gave rise to the doublets in Fig. 2, lanes 6–9, was investigated by PE of RNAs that were treated with tobacco acid pyrophosphatase (TAP). For each doublet, TAP treatment reduced the intensity of the upper band and increased that of the lower band, regardless of whether the initial source of RNA3 was an RNA3 transcript or RNA1 (data not shown). TAP treatment had no effect on the PE products corresponding to T7 transcripts. These results indicated that some fraction of the replicated RNA3 products were capped.

The uncapped replication product from FHV3(G,0) gave rise to a PE product that migrated 1 nt more rapidly than that from the predominant T7 transcript (Fig. 2, compare lanes 7 and 3). This result, and the comigration of PE products that corresponded to replication products from FHV3(G,0) and those produced during replication of RNA1 (Fig. 2, compare lanes 7 and 9), indicated that the 5′ end was corrected during RNA3 replication. Furthermore, when cells were transfected with 3VHF(G,0), a transcription plasmid that expressed (−)-sense RNA3, PE yielded an RdRp-dependent doublet that comigrated with those from (+)-sense replicons (Fig. 2, lane 11). This result showed that the RdRp used the (−)-sense T7 transcript as a template to produce (+)-sense RNA3 with a correct 5′ end (also see below).

Removal of the extra G from the 5′ end of (+)-sense transcripts was independently analyzed by sequencing of bulk RT-PCR products amplified from (−)-sense RNA3 dimer junctions as described in Albariño et al. (2001). Cells were transfected with plasmids expressing (+)-sense RNA3 with no extra nucleotide, or with an extra G, GC, or GG at the 5′ end in the absence or presence of pTMFA. No dimer junction-specific RT-PCR products were detected from cells that received only the RdRp or only an RNA3 plasmid (data not shown). However, upon cotransfection with the RdRp and an RNA3 plasmid, the predominant junction sequence was the same regardless of the nonviral sequence at the 5′ end of the RNA3 transcripts: a perfect head-to-tail junction (5′. . .AGGT|GTTA. . .3′ in the (+)-sense) that contained no inserted or deleted nucleotide(s). The absence of extra residues from the 5′ end of T7 transcripts in the majority of RNA3 dimer junctions, and their absence at the 5′ end of RNA3 replication products as determined by PE (Fig. 2), indicates that the sequence at the 5′ end of RNA3 was corrected in an RdRp-dependent manner. A minor fraction of RNA3 dimer junctions from FHV3(GC,0) contained an extra C residue (in the (+)-sense) at the junction corresponding to the second of the two additional nucleotides at the 5′ end of the T7 transcript (data not shown). Nonviral residues from the 5′ end of T7 transcripts of RNA1 and RNA3 have previously been detected in RNA1 and RNA3 homodimers, respectively (Albariño et al., 2003, 2001).

Replication of Pariacoto virus (PaV) RNA3

To determine whether the ability to replicate RNA3 was unique to FHV, we developed a complementation system to study RNA replication for another nodavirus, *Pariacoto virus.* A PaV protein A expression plasmid (pTMPA) analogous to pTMFA was constructed to provide the PaV RdRp. As a control, a plasmid for transcription of an ORF A frame-shifted PaV RNA1 template was also constructed [PaV1(1,0)AKO]. Cells transfected with either of these plasmids individually showed no detectable PaV RNA1 or RNA3 replication products when subjected to Northern blot analysis (Fig. 3, lanes 3 and 4). On cotransfection of the two plasmids, RNA1 and RNA3 were detected (Fig. 3, lane 6), indicative of RNA1 replication. We also detected several

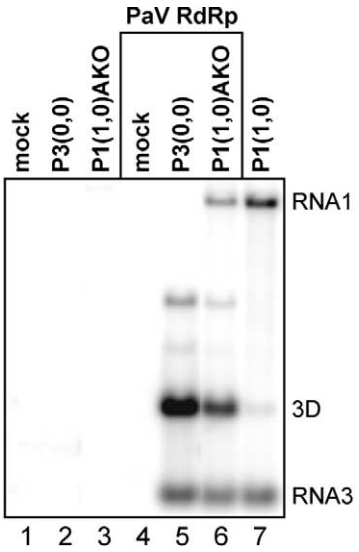

Fig. 3. Northern blot analysis of PaV RNA3 replication products. Cells were mock transfected (lane 1) or transfected with plasmids expressing either full-length PaV RNA3 [PaV3(0,0)] or full-length PaV RNA1 in which ORF A was frame-shifted to create a premature termination codon [PaV1(1,0)AKO] alone (lanes 2 and 3) or together with a plasmid expressing PaV protein A (pTMPA) to provide the viral RdRp (lanes 5 and 6). PaV RNA3 and RNA1 are abbreviated as P3 and P1, respectively, in the plasmid names in the figure. Lane 4 corresponds to cells that received pTMPA alone. For comparison, cells were transfected with PaV1(1,0), a plasmid for transcription of autonomously replicating wild-type PaV RNA1, as a source of authentic RNA3 (lane 7). Total RNA was resolved and transferred as in Fig. 1B. Blots were hybridized with an RNA probe complementary to (+)-sense PaV RNA3. RNAs 1 and 3 are indicated, as are homodimers of RNA3 (3D).

RNAs that we interpreted as a series of RNA3 oligomers: homodimers, -trimers, and -tetramers. The presence of RNA3 dimers was confirmed by DNA sequence analysis of an RT-PCR product from the sample shown in Fig. 3, lane 6 (data not shown). RNA1 and monomers and dimers of RNA3 comigrated with those produced during replication of the autonomously replicating wild-type RNA1 transcripts from plasmid PaV1(1,0) (Fig. 3, lane 7; Johnson et al., 2000). Unexpectedly, the Northern blot data showed that PaV RNA3 dimers were more abundant than monomers when the RdRp was provided *in trans.*

When cells were cotransfected with PaV3(0,0), a T7 transcription plasmid expressing PaV RNA3, and pTMPA to provide the PaV RdRp *in trans*, the pattern of RNA3 species was similar to that from the frame-shifted RNA1 template (Fig. 3, compare lanes 5 and 6). In the absence of the RdRp, the DNA-templated primary transcripts from PaV3(0,0) were undetectable at this level of sensitivity (Fig. 3, lane 2). These results strongly suggested that, similar to the FHV sgRNA, PaV RNA3 could be replicated in the absence of a fully functional RNA1 template. This conclusion was confirmed both by Northern blot detection of (−)-sense RNA3 species and by metabolic labeling of RNAs in the presence of Act D (data not shown). A similar ratio of RNA3 monomer to dimer was observed by labeling as by Northern blot hybridization, indicating that the accumulated levels of these RNA3 species approximated their relative rates of synthesis. Furthermore, closing the B1 and B2 ORFs did not significantly affect the replication of PaV RNA3, indicating that neither of these proteins was required for RNA3 replication (data not shown). Together, these data indicate that both FHV and PaV RNA3 can be replicated by their cognate RdRps. However, cross-replication of the sgRNA of one virus by the RdRp of the other was undetectable at this level of sensitivity, indicating that the viral RdRps have distinct template specificities (data not shown). This result was not surprising in view of the overall divergence of the two protein A sequences and the different RNA termini of these two viruses (Johnson et al., 2001, 2000).

Replication of FHV RNA3 dimers

RNA3 homodimers accumulated during replication of FHV or PaV RNA3 replicons and during RNA3 synthesis from replicating RNA1 (Figs. 1B and 3). To examine the template properties of FHV RNA3 dimers, we constructed T7 transcription plasmids expressing (+)-sense dimeric RNA3 transcripts. Because FHV RNA3 dimer junctions recovered from RNA3 replicons lacked any inserted or deleted nucleotides, whereas those recovered during RNA1 replication contained an extra C residue (in the (+)-sense), we constructed plasmids to transcribe RNA3 dimers with either type of junction, as illustrated schematically in Fig. 4A. Cells were transfected with either of these plasmids individually or together with pTMFA to provide the FHV RdRp *in trans*, and the (+)-sense products of transcription and replication were detected by Northern blot hybridization.

As with the monomeric transcripts (Fig. 4B, lane 1), the major T7 dimeric transcripts had the mobility expected for ribozyme-uncleaved species (UCDT) (Fig. 4B, lanes 2 and 3). In the presence of RdRp transcripts, both dimeric transcripts predominantly templated replication of RNA3 dimers (Fig. 4B, lanes 6 and 7). Interestingly, the single-nucleotide difference at the homodimer junction led to a small but reproducible effect. Dimers without the extra C residue at the junction yielded low but readily detectable levels of monomeric RNA3 (Fig. 4B, lane 6), whereas those with the extra C, which resemble the RNA3 homodimers detected during replication of wild-type FHV in cultured *Drosophila* cells (Albariño et al., 2001), produced barely detectable monomer (Fig. 4B, lane 7). In either case, the resolution of monomers from transcribed or replicated RNA3 homodimers was poor, in contrast to the predominant replication of RNA2 monomers templated by homodimers of FHV RNA2 (Albariño et al., 2001).

In addition to RNA3 monomers and dimers, the replication products contained two other minor RNA species: a putative RNA3 tetramer (the slowest migrating RNA in Fig. 4B, lanes 6 and 7), and a species that migrated slightly faster than the RNA3 dimer (particularly prominent in Fig. 4B,

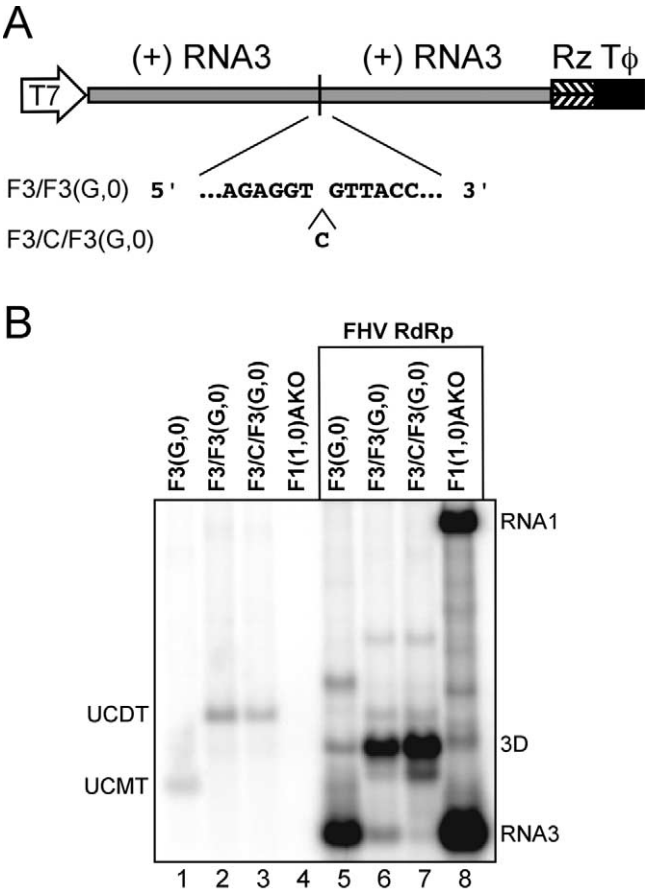

Fig. 4. Replication of FHV RNA3 dimers. (A) Schematic of a plasmid for transcription of (+)-sense dimeric FHV RNA3. Two such plasmids were constructed that differed only in the sequence at the dimer junction. In plasmid FHV3/FHV3(G,0) the 3′-terminal sequence of the upstream copy immediately preceded the 5′-terminal sequence of the downstream copy, whereas in plasmid FHV3/C/FHV3(G,0) the two cDNA copies were separated by a single C residue. (B) Northern blot analysis of replication products of RNA3 dimers. Cells were transfected with FHV3(G,0), FHV3/FHV3(G,0), FHV3/C/FHV3(G,0), or FHV1(1,0)AKO alone (lanes 1–4) or together with pTMFA (lanes 5–8). Total RNA was analyzed by Northern blot hybridization as in Fig. 1B. UCMT and UCDT indicate uncleaved monomeric and dimeric primary transcripts, respectively, from the RNA3 plasmids. RNAs 1 and 3 and RNA3 homodimers (3D) are also indicated.

lane 7). The identity of the latter RNA is unknown, but as it does not hybridize to oligodeoxynucleotides complementary to either the T7 promoter or the 5′-terminal region of the HDV ribozyme (data not shown), it may be an internally deleted version of the RNA3 dimer that arose during RNA replication.

Synthesis of positive-sense RNA3 from negative-sense templates

Previous studies in our laboratory have shown that FHV RNA2 replication can be initiated from (−)-sense T7 transcripts transcribed intracellularly (Albariño et al., 2001; Ball, 1994). Since (−)-sense RNA3 is produced during FHV infection (Zhong and Rueckert, 1993) and RNA replication initiated from plasmids (Eckerle and Ball, 2002; Price et al., 2000), we examined whether (−)-sense templates could be used to initiate RNA3 replication. T7 transcription plasmids 3VHF(G,0) and 3VHF/3VHF(G,0) were developed to express (−)-sense monomers and dimers of FHV RNA3, respectively, as illustrated schematically in Fig. 5A. As for the (+)-sense RNA3 dimer transcription plasmids, a second (−)-sense RNA3 dimer plasmid, 3VHF/G/3VHF(G,0), was constructed that differed only by the presence of an extra nucleotide (G in the (−)-sense) at the dimer junction.

These plasmids were transfected individually or together with pTMFA to provide the FHV RdRp. Northern blot analysis of the transcription and replication products was performed using strand-specific oligodeoxynucleotide probes (see Materials and methods). Hybridization with a probe complementary to (−)-sense RNA3 showed that the ribozyme-uncleaved and cleaved (−)-sense T7 transcripts migrated as expected (data not shown). RdRp-dependent (+)-sense products were detected using a probe complementary to (+)-sense RNA3 (Fig. 5B, lanes 6–10). In the presence of RdRp, the predominant (+)-sense RNA product templated by 3VHF(G,0) transcripts comigrated with RNA3 produced during replication of (+)-sense transcripts from FHV3(G,0) (Fig. 5B, compare lanes 6 and 9). However, this product was ~15-fold less abundant when templated by transcripts from 3VHF(G,0) than when RNA3 replication was initiated by (+)-sense transcripts from FHV3(G,0) (Fig. 5B, lane 10). The (−)-sense monomeric transcripts from 3VHF(G,0) apparently also templated (+)-sense dimers (Fig. 5B, lane 6) in ~15-fold lower abundance than those from the (+)-sense construct FHV3(G,0). Two minor RNA species that migrated between RNA3 monomer and dimer were detected among the (+)-sense RNA products from 3VHF(G,0) + pTMFA (Fig. 5B, lane 6). The identities of these minor RNA3-related species remain to be determined, although they did not hybridize with oligodeoxynucleotides complementary to either the T7 promoter or the HDV ribozyme (data not shown).

Surprisingly, although (−)-sense dimers templated the synthesis of (+)-sense dimers, no monomers were detected (Fig. 5B, lanes 7 and 8). Similar to their (+)-sense counterparts, slightly more RNA3 dimers were made from transcripts with the extra nucleotide at the dimer junction than without it. This result was notable in that the presence of a single nucleotide in the middle of a 774-nt RNA affected its replication. When the authentic (−)-sense junction G residue was changed to a C in a (−)-sense dimeric primary transcript, the RdRp-dependent products were indistinguishable from that without an extra nucleotide (data not shown). These results show that although the junction sequences can affect the template properties of RNA3 homodimers, some alternative junction sequences can be tolerated.

Regardless of whether the (−)-sense transcripts were

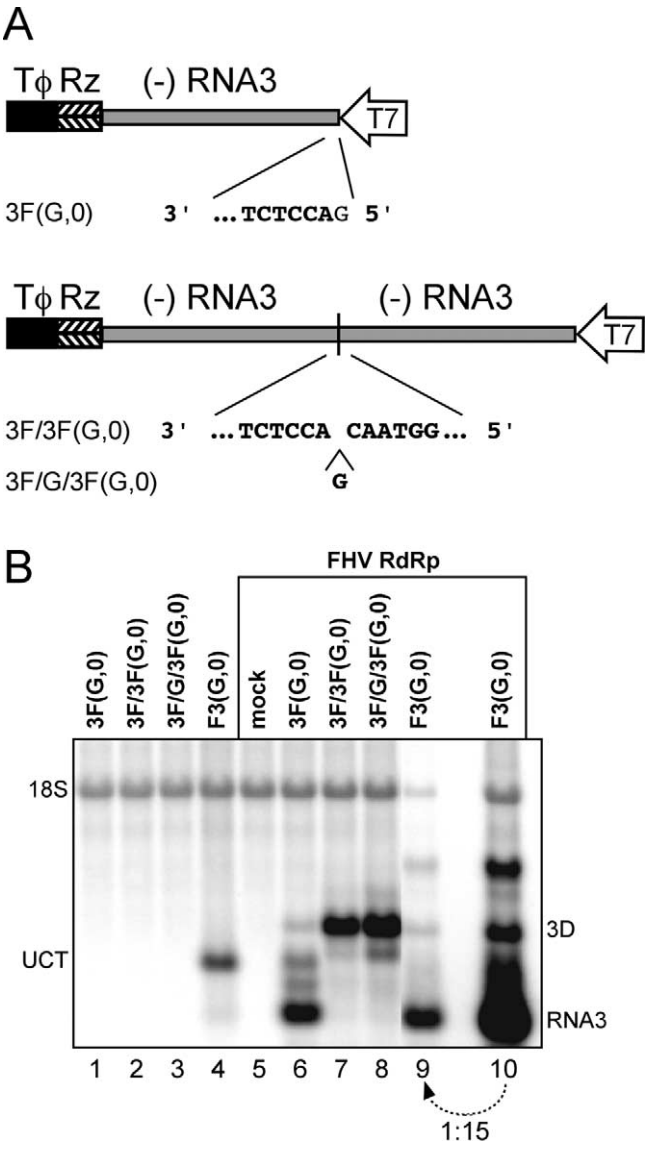

Fig. 5. Positive-sense RdRp-dependent products from negative-sense monomeric and dimeric FHV RNA3 transcripts. (A) Schematics of plasmids for transcription of (-)sense monomeric and dimeric RNA3, 3VHF(G,0), and 3VHF/3VHF(G,0), respectively. 3VHF is abbreviated as 3F in the figure. The nonviral G residue preceding the (−)-sense RNA3 cDNA in 3VHF(G,0) is shown in plain type. Plasmid 3VHF/G/3VHF(G,0) expresses (−)-sense dimeric RNA3 with an extra G residue at the dimer junction relative to that from plasmid 3VHF/3VHF(G,0). (B) Northern blot analysis of RdRp-dependent products from (−)-sense RNA3 transcripts. Cells were transfected with the indicated (−)-sense RNA3 transcription plasmids alone (lanes 1–3) or together with pTMFA (lanes 6–8). Lane 5 corresponds to cells that received pTMFA alone. As controls, cells were transfected with the (+)-sense RNA3 transcription plasmid FHV3(G,0) alone (lane 4) or together with pTMFA (lanes 9 and 10). Total RNA was analyzed by Northern blot hybridization to detect (+)-sense FHV RNA3 as in Fig. 1B except that an oligodeoxynucleotide probe was used for enhanced strand-specificity. This probe showed some cross-reactivity with cellular 18S rRNA, as indicated. The RNA sample loaded in lanes 9 and 10 was the same except that 1/15 as much was loaded in lane 9. UCT indicates uncleaved primary transcripts from FHV3(G,0). RNA3 monomers and homodimers (3D) are also indicated.

monomers or dimers, no RdRp-dependent (−)-sense RNA3 species were detected (data not shown). Together, these data imply that (−)-sense RNA3 primary transcripts undergo only half of the replication cycle, from (−)- to (+)-sense, but not vice versa. Engineered removal of the nonviral G residue from the 5′ end of (−)-sense transcripts did not alleviate this block (data not shown).

Transactivation of FHV RNA2 replication correlated with replication of RNA3

We showed previously that to replicate RNA2, the FHV RdRp required RNA3 supplied either by replication of an RNA1 template or from an RNA3 transcription plasmid (Albariño et al., 2003; Eckerle and Ball, 2002). Having established in the current study that RNA3 replicated fully from (+)-sense transcripts (Figs. 1B and C), we next examined the relationship between RNA3 replication and transactivation of RNA2 replication. We had available three variants of FHV RNA1 cDNA having insertions or deletions in the 3′ UTR that prevented RNA1 replication and RNA3 synthesis as assayed by metabolic RNA labeling and Northern blot analysis (data not shown). To determine the effect of these mutations on RNA3 replication, they were transferred into plasmid FHV3(G,0). As shown schematically in Fig. 6A, they included insertion of 6 nt between the last nucleotide of the ORF B2 stop codon (RNA3 nt 338) and the first nucleotide of the 3′ UTR (plasmid FHV3(G,0)+6); deletion of 8 nt (RNA3 nt 347–354) (plasmid FHV3(G,0)Δ8); and deletion of the entire 49-nt 3′ UTR (plasmid FHV3(G, −49)). Cells were transfected with each of these RNA3 plasmids together with pTMFA to provide the RdRp, and the RNA products of transcription and replication were analyzed by Northern blot hybridization as before. Plasmids FHV3(G,0), FHV3(G,0)B1/B2KO, and FHV1(1,0)AKO yielded the expected products of RNA replication (Fig. 6B, lanes 1, 2, and 6), whereas the 3′ UTR mutant plasmids yielded only the DNA-templated primary transcripts (largely uncleaved by the HDV ribozyme) (Fig. 6B, lanes 3–5). These results indicated that each of the three mutations eliminated replication of RNA3 as they had that of RNA1.

When the RdRp was supplied with RNA2 transcripts from plasmid FHV2(0,0) in the absence of RNA3, no RNA2 replication occurred (Fig. 6B, lane 7), as described before (Eckerle and Ball, 2002). RNA3 from wild-type or B1/B2-deficient replicons or from ORF A deficient RNA1 transactivated robust RNA2 replication, as evidenced by the accumulation of RNA2 monomers and homodimers (Fig. 6B, lanes 8, 9, and 13). In each of these cases, RNA3 accumulation was strongly suppressed by RNA2 replication, as occurs during FHV infection of cultured *Drosophila* cells (Gallagher et al., 1983). In contrast, each of the three nonreplicating RNA3 variants failed to transactivate detectable RNA2 replication (Fig. 6B, lanes 10–12), establishing a

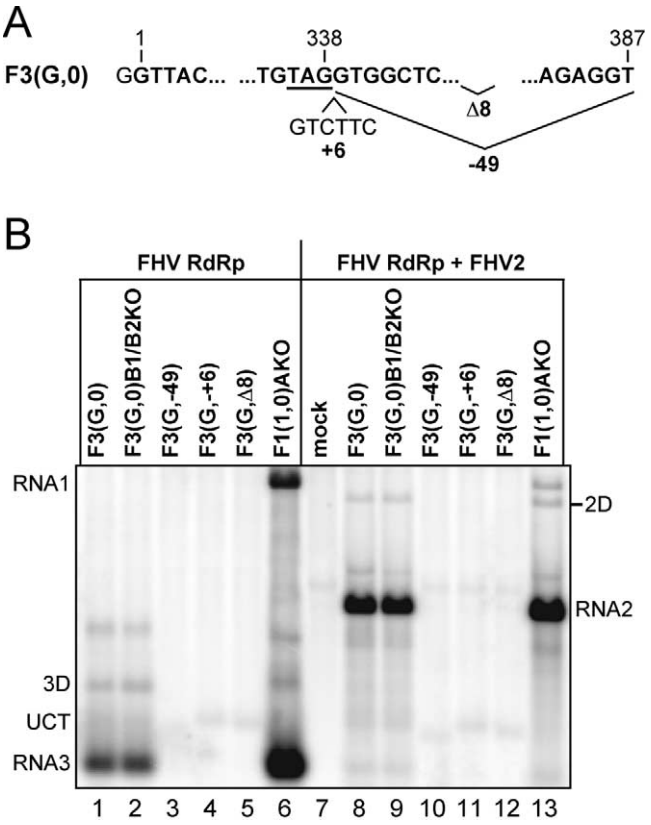

Fig. 6. Transactivation of FHV RNA2 replication by RNA3 replicons. (A) Schematic of insertion and deletion mutants of FHV RNA3. A partial sequence of the RNA3 cDNA in plasmid FHV3(G,0) is shown. Three transcription plasmids that express insertion or deletion mutants of (+)-sense FHV RNA3 were constructed: FHV3(G,0)+6 contained an insertion of 6 nt immediately downstream of the ORF B2 stop codon (underlined), FHV3(G,0)Δ8 lacked RNA3 nt 348–355, and FHV3(G,−49) lacked precisely the entire 3′ UTR. (B) Northern blot analysis of FHV RNA replication products. Cells were transfected with RNA3 transcription plasmids expressing wild-type, B1/B2KO, or the three mutants mentioned above together with pTMFA (lanes 1–5) or together with pTMFA and FHV2(0,0), a plasmid for transcription of RNA2 (lanes 8–12). Cells in lane 7 received pTMFA and FHV2(0,0) but no RNA3 plasmid. As controls, cells were transfected with FHV1(1,0)AKO and pTMFA (lane 6) or with these two plasmids and FHV2(0,0) (lane 13). Total RNA was analyzed by Northern blot hybridization as in Fig. 1B except that the blot was hybridized simultaneously with RNA probes complementary to (+)-sense RNA3 and RNA2. RNAs 1, 2, and 3 are indicated, as are homodimers of RNA3 (3D) and RNA2 (2D). UCT indicates the uncleaved primary transcripts from the RNA3 plasmids.

correlation between replication of RNA3 and its ability to transactivate RNA2.

Mapping the cis-acting replication element at the 3′ end of RNA3

In a recent study we replaced a central region of RNA1 and RNA2 with a heterologous sequence and were thereby able to define the approximate 5′ boundary of the minimal 3′-terminal *cis*-acting sequences required for replication (Albariño et al., 2003). For RNA1 this boundary lay between 48 and 108 nt upstream of the 3′ end. To compare the minimal 3′-terminal sequence necessary for RNA3 replication with that of RNA1, we applied a similar strategy to the RNA3 replicon FHV3(G,0). A core sequence of 728 nt derived from the enhanced yellow fluorescent protein (eYFP) gene was inserted into RNA3 in the antisense orientation relative to the flanking regions to generate an 1120-nt replicon containing RNA3-derived 5′ and 3′ termini of 86 and 306 nt, respectively (plasmid $F3eF3_{306}$). We progressively shortened the 3′-terminal RNA3 segment to leave the terminal 192, 108, 58, and 48 nt from the 3′ end of RNA3 in constructs $F3eF3_{192}$, $F3eF3_{108}$, $F3eF3_{58}$, and $F3eF3_{48}$, respectively (Fig. 7). Each of these RNA3 replicon plasmids was transfected into cells together with pTMFA, and RNA products were analyzed by Northern blot hybridization using a probe complementary to a portion of the 5′-terminal 86 nt of (+)-sense RNA3 common to the replicons illustrated in Fig. 7.

Replication products of the expected sizes were observed for RNA3 derivatives $F3eF3_{306}$, $F3eF3_{192}$, $F3eF3_{108}$, and $F3eF3_{58}$, but not for $F3eF3_{48}$ (Fig. 7, lanes 1–5). These results showed that when flanked by the termini of RNA3, the eYFP core was tolerated by the FHV RdRp, as we have shown previously for RNA1 and RNA2 contexts (Albariño et al., 2003). Furthermore, these results mapped the upstream boundary of the 3′ *cis*-acting signal necessary for RNA3 replication to between 48 and 58 nt from the 3′ end of RNA3 (Fig. 7, lanes 4 and 5). Since RNAs 1 and 3 are 3′ coterminal, we tested the same 3′-terminal 48- and 58-nt regions for their abilities to support the replication of an analogous eYFP-containing replicon that contained the 5′-terminal 297 nt from RNA1. As with the RNA3 replicon, the 3′-terminal 58 nt supported replication of the RNA1 derivative, but the 3′-terminal 48 nt did not (data not shown).

To determine whether the F3eF3 replicons were able to transactivate RNA2 replication, the five F3eF3 constructs and FHV3(G,0) were each transfected together with pTMFA and FHV2(0,0) to provide FHV RdRp and RNA2, respectively, and a probe complementary to (+)-sense RNA2 was added for the Northern blot analysis. As before, the ability to transactivate replication of RNA2 correlated with the ability of the RNA3 derivatives to replicate (Fig. 7, lanes 8–13). The four F3eF3 constructs that replicated resulted in accumulation of RNA2 monomers and dimers (Fig. 7, lanes 8–11), whereas no RNA2-specific products were produced in response to $F3eF3_{48}$, which itself failed to replicate (Fig. 7, lanes 5 and 12). Together, these results indicate that replicable F3eF3 derivatives transactivated RNA2, whereas the nonreplicable one failed to do so. In the presence of RNA2, the F3eF3 replicons accumulated to higher levels than RNA3 transcribed from FHV3(G,0) and, maybe as a consequence, resulted in slightly higher levels of RNA2 (Fig. 7, compare lanes 8–11 and 13). Since the F3eF3 transcripts were not detectable in the absence of replication (see Fig. 7, lanes 5 and 12), these results suggest

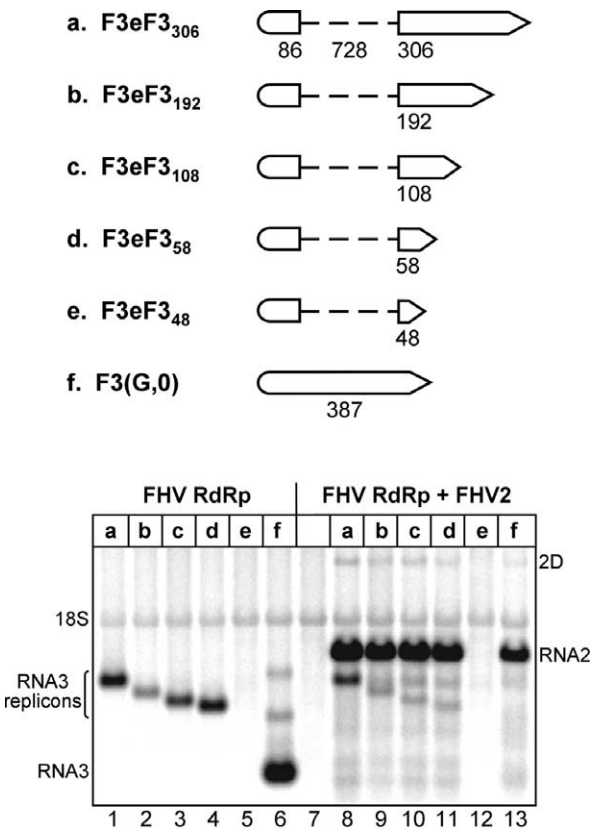

Fig. 7. Replication of FHV RNA3 derivatives that share a common heterologous central core, and transactivation of RNA2. FHV RNA3-derived replicons were expressed from T7 transcription plasmids. Schematics of each replicon are shown at the top. The terminal sequences derived from (+)-sense RNA3 are indicated by F3 in the replicon names and by rounded and pointed boxes representing the 5′ and 3′ termini, respectively, in the schematic. The central core (dashed line) contains 728 nt derived from the eYFP gene (e) in the antisense orientation relative to the flanking sequences. Numbers below each segment indicate its length in nucleotides. Lengths of 3′-terminal segments are also indicated by subscripts in the replicon names. Letters preceding each replicon name indicate the corresponding lane of the Northern blot. Cells were transfected with the indicated replicons (a–f) together with pTMFA either without (lanes 1–6) or with FHV2(0,0) (lanes 8–13). Cells in lane 7 received pTMFA and FHV2(0,0) but no RNA3 replicon. Total RNA was analyzed by Northern blot hybridization as in Fig. 1B except that the blots were simultaneously hybridized to oligodeoxynucleotides complementary to a portion of the (+)-sense 5′-terminal segment common to all RNA3 replicons and to (+)-sense RNA2. RNA replication products corresponding to the RNA3 replicons are indicated. 18S rRNA, RNAs 2 and 3, and RNA2 homodimers (2D) are also indicated.

that the F3eF3 replicons were less sensitive to RNA2-mediated suppression than RNA3 itself.

Discussion

In the current work we directly tested the possibility that nodaviral RNA3 can be replicated by the viral RdRp. By reconstructing RNA replication in plasmid-transfected cells in the absence of replicating RNA1, we demonstrated that (+)-sense RNA3 transcripts templated the synthesis of (−)-sense intermediates, which in turn templated (+)-strand RNA3 synthesis, thereby establishing full RNA3 replication (Figs. 1B and C). RdRp-dependent correction of 5′ nonviral residues provided further evidence that the RdRp used RNA3 transcripts as replication templates (Fig. 2). Since the only available RNA3 template in these experiments was the plasmid transcript, these results eliminated the ambiguity of the natural situation in which both RNA1 and RNA3 templates may contribute to RNA3 synthesis. The ability of the RdRp to fully replicate the sgRNA extended to another nodavirus, PaV (Fig. 3), although the FHV and PaV RdRps did not replicate each other's RNA3. The results reported here for FHV and PaV constitute the first direct evidence that the nodaviral RdRp can catalyze full sgRNA replication. The question remains open as to whether sgRNAs of other (+)-strand RNA viruses replicate fully. Candidates for sgRNA replication include those viruses for which it is thought that the first subgenomic-length RNAs synthesized are of (−) polarity (Sawicki and Sawicki, 1998; White, 2002).

Viral protein requirements for RNA3 replication and transactivation

It was demonstrated previously that the RNA3-encoded proteins B1 and B2 are not required for FHV RNA1 replication (Ball, 1995) or for RNA2 transactivation when RNA3 is provided from a functional RNA1 template (Eckerle and Ball, 2002). Similarly, the current results showed that these proteins were dispensable for replication of RNA3 (Fig. 1B) and for transactivation of RNA2 by RNA3 replicons (Fig. 6). Because the capsid proteins are also dispensable for RNA2 replication (Zhong and Rueckert, 1993), the only known viral protein required for transactivation is protein A itself.

RNA3 3′ replication signals

We used two independent approaches to investigate the replication signals at the 3′ end of RNA3: making deletions in the 3′ segments of RNA3 transcripts that contained a common eYFP core, and making deletions or insertions toward the 3′ end of otherwise wild-type RNA3 transcripts. We recently used the former approach to define the 5′ boundary of the minimal 3′-terminal *cis*-acting sequences required for RNA1 and RNA2 replication (Albariño et al., 2003). Extending this approach to RNA3 showed that the 3′-terminal 58 nt were sufficient to support replication, whereas 48 nt were not (Fig. 7). Identical results were obtained for RNA1, suggesting that the minimal 3′ signal may be the same for the two RNAs. Deletion of the entire 49-nt 3′ UTR from otherwise wild-type RNA3 prevented replication, as did a 6-nt insertion or an 8-nt deletion in this region, indicating that the 3′ UTR was rather intolerant of change at these positions (Fig. 6). However, inserting ~700

nt of foreign sequence 70 nt upstream of the 3′ end of RNA1 did not prevent either RNA1 replication or RNA3 synthesis (Price et al., 2000).

Positive-sense RNA3 synthesis from negative-sense RNA3 templates

We demonstrated previously that autonomously replicating FHV RNA1 supports the synthesis of (+)-sense RNA2 from (−)-sense transcripts (Albariño et al., 2001; Ball, 1994). Here, we showed that in the absence of a replication-competent RNA1 template the FHV RdRp synthesized (+)-sense RNA3 from (−)-sense RNA3 transcripts (Fig. 5) and that the (+)-sense RNA3 progeny had 5′ ends identical to those of authentic RNA3 (Fig. 2). This step of copying a (−)-sense sgRNA into its (+)-sense complement is central to the premature termination model of sgRNA synthesis, which has been proposed for several different (+)-strand RNA viruses (reviewed in White, 2002). While these data fulfill a critical requirement of the premature termination model, they are not incompatible with the alternative model for RNA3 synthesis: internal initiation on the RNA1 (−)-strand.

Despite the synthesis of (+)-sense products from (−)-sense RNA3 transcripts, there was no measurable difference in the levels of (−)-sense RNA3 in the absence or presence of the RdRp. These results suggest that (−)-sense RNA3 transcripts were only hemireplicated (i.e., copied from (−) to (+)). We cannot rule out the possibility that the block to (−)-strand synthesis is due to a technical aspect of the plasmid-driven experimental system, but an alternative explanation is that (+)-sense RNA3 progeny are transported from replication complexes to an intracellular compartment that is inaccessible to the RdRp, as suggested for replication of *Brome mosaic virus* RNA by Schwartz et al. (2002). Furthermore, the different template properties of (+)-sense and (−)-sense RNA3 transcripts raise the question of whether RNA3 undergoes hemi- or full replication when initially templated by RNA1, and of the relative contributions of sgRNA replication and RNA1-templated transcription to the overall synthesis of RNA3. Further studies will be necessary to answer these questions.

Comparison of the template properties of RNA2 and RNA3 homodimers

Our results showed that both RNAs 2 and 3 generated homodimers during replication of monomeric templates (Fig. 6; Albariño et al., 2001), although the significance of RNA dimers in general remains unclear. Whatever their role, homodimers of RNAs 2 and 3 have different template properties: whereas RNA2 homodimers of either polarity were readily resolved by the RdRp into monomers (Albariño et al., 2001), (+)-sense RNA3 homodimers were resolved poorly (Fig. 4). (−)-sense RNA3 homodimers remained unresolved and were copied into (+)-sense dimers only (Fig. 5).

Homodimers of RNAs 2 and 3 also differed in the effects of specific sequences at the dimer junctions. The major species of RNA2 homodimer lacked one copy of the terminally redundant GU dinucleotide, whereas a minor species contained both GU copies intact. The template properties of T7 transcripts of the two species were indistinguishable (Albariño et al., 2001). Similarly, RNA3-RNA2 heterodimers with . . .GU. . . or . . .GUGU. . . junctions yielded identical replication products (Albariño et al., 2003). In contrast, RNA3 homodimers with the naturally occurring extra residue at the junction replicated slightly better than those without it (or with an unnatural junction nucleotide), although they resolved into monomers less well (Figs. 4 and 5).

Why was the resolution of RNA3 dimers so poor? We currently think that FHV RNA dimers are resolved into monomers by two pathways: as a result of an RdRp jump from one monomeric component of the dimer template to the other component during homologous recombination, and as a result of the RdRp responding to internal *cis*-acting signals located at the dimer junction on the template (Albariño et al., 2001). In accordance with the former pathway, the low resolution of homodimers of RNA3 relative to those of RNA2 may be explained by the smaller target size of RNA3. This explanation is supported by the observation that dimers of a 236-nt derivative of RNA2 with most of the internal sequence deleted were resolved poorly, if at all, into monomers (data not shown).

Counterregulation of the synthesis of RNAs 2 and 3

We demonstrated previously that FHV RNA3 is required to transactivate RNA2 replication (Albariño et al., 2003; Eckerle and Ball, 2002). In the current work, we found that mutations that prevented RNA3 replication also prevented transactivation (Figs. 6 and 7). To date, only RNA3 derivatives that can replicate can transactivate RNA2, suggesting that replication of RNA3 may be necessary for transactivation. Further studies are underway to compare the RNA3 sequence requirements for replication and transactivation.

RNA2 replication suppressed the accumulation of RNA3 not only in the context of transcription from an RNA1 template, but also when RNA3 replication was initiated from T7 RNA3 transcripts (Figs. 6 and 7). This is the first evidence that RNA2 replication can suppress RNA3 replication. The observation that RNA3 replicons that contained eYFP sequences supported higher levels of RNA2 replication than the wild-type transcripts from FHV3(G,0) may perhaps be explained by their greater resistance to RNA2-mediated suppression (Fig. 7). This explanation would be consistent with the proposal that a dynamic counterregulation between RNAs 2 and 3 coordinates the replication of the two viral genome segments (Eckerle and Ball, 2002).

Materials and methods

Transcription plasmids

Plasmids were generated using standard methods of DNA manipulation (Sambrook and Russell, 2001). For PCR mutagenesis, small PCR-generated DNA fragments were substituted into parental plasmids and the transferred sequences were verified. The FHV protein A expression plasmid pTMFA has been described (Eckerle and Ball, 2002). A comparable plasmid (pTMPA) for expression of PaV protein A was constructed in the same manner using the pTM1 backbone (Moss et al., 1990). pTMPA contained the wild-type nucleotide sequence of PaV ORF A.

T7 transcription plasmids that expressed (+)-sense FHV RNA1 and RNA2 [FHV1(1,0) and FHV2(0,0), respectively] or PaV RNA1 [PaV1(1,0)] have been described (Ball, 1995; Ball and Li, 1993; Johnson and Ball, 2001). These plasmids contained full-length cDNA of the respective viral RNA positioned immediately downstream of a T7 promoter and immediately upstream of the self-cleaving antigenomic ribozyme from HDV (Perrotta and Been, 1991). A T7 terminator (Rosenberg et al., 1987) followed the ribozyme. The number of nonviral nucleotides present at the 5′ and 3′ termini of transcripts after ribozyme cleavage are indicated in parentheses (*x*, *y*).

Plasmids FHV1(1,0)AKO and PaV1(1,0)AKO were designed for transcription of full-length FHV or PaV RNA1 templates, respectively, that could not express functional protein A. ORF A was frameshifted by cleavage at the unique *Eag*l site (FHV) or *Nde*l site (PaV), and the DNA termini were filled in with Klenow DNA polymerase (Invitrogen Life Technologies) followed by ligation with T4 DNA ligase (Invitrogen Life Technologies). For FHV1(1,0)AKO, the start codon of ORF A was also changed from ATG to ATC (G42C).

T7 transcription plasmids that expressed (+)-sense wild-type FHV RNA3 with the indicated additional nucleotides at the 5′ end included FHV3(0,0), FHV3(G,0), FHV3(GC,0), and FHV3(GTC,0). Transcripts of FHV3(G,0)B1/B2KO contained two mutations (U9A and U19A) that changed the start codons of ORFs B1 and B2 from AUG to AAG as well as a third mutation (C190A) that introduced a premature stop codon in ORF B2 after 57 codons. Transcripts of FHV3(G,−49) lacked the entire 49-nt RNA3 3′ UTR (RNA3 nt 339–387), whereas those of FHV3(G,0)Δ8 lacked the 8 nt from RNA3 nt 348–355 and those of FHV3(G,0)+6 contained an insertion of a 6-nt *Bbs*l restriction site between RNA3 nt 338 and 339. To generate (−)-sense RNA3 transcripts, full-length cDNA of FHV RNA3 was cloned in inverted orientation between the T7 promoter and the ribozyme, resulting in plasmid 3VHF(G,0). An extra G residue was included at the 5′ end to satisfy the requirements for transcription by T7 RNA polymerase.

We also constructed plasmids that transcribed FHV RNA3 dimers by inserting a second copy of the FHV RNA3 cDNA between the first copy and the ribozyme. Cleaved transcripts of plasmids FHV3/FHV3(G,0) and 3VHF/3VHF(G,0) consisted of (+)- or (−)-sense dimeric RNA3, respectively, with no insertion or deletion at the dimer junction. Transcripts of FHV3/C/FHV3(G,0) and 3VHF/G/3VHF(G,0) contained a single extra nucleotide between the two RNA3 monomers as indicated in the plasmid names. PaV3(0,0) was constructed such that its cleaved transcripts would contain exactly the reported sequence of PaV RNA3 (PaV RNA1 nt 2598–3011; Johnson et al., 2000).

We replaced the central sequences of FHV RNA3 with a central core consisting of 728 nt containing the entire ORF of the enhanced yellow fluorescent protein (from pIRES-EYFP, Clontech) in the antisense orientation with respect to the T7 promoter and flanking FHV sequences, as described previously (Albariño et al., 2003). Plasmids expressing these RNA3 replicons (F3eF3 derivatives) contained 86 nt (RNA3 nt 1–86) from the 5′ end of RNA3 followed by the same eYFP central core and the terminal 306 (nt 82–387), 192 (nt 196–387), 108 (nt 280–387), 58 (nt 330–387), or 48 nt (nt 340–387) from the 3′ end of RNA3. Each F3eF3 plasmid had one extra G residue between the T7 promoter and the FHV cDNA to facilitate transcription by T7 RNA polymerase. The RNA3-derived sequence in $F3eF3_{306}$ (86 + 306 = 392 nt) was 5 nt longer than the 387 nt of wild-type RNA3 because of a duplication introduced during construction.

Cells and plasmid transfection

The maintenance of BSR-T7/5 cells (Buchholz et al., 1999) and their transfection with plasmid DNA have been described (Eckerle and Ball, 2002). Cells in 35-mm-diameter tissue culture wells were transfected with 1 μg of pTMFA or pTMPA, 5 μg of each RNA3 transcription plasmid that lacked the eYFP sequence, 4 μg of each F3eF3 plasmid, or 2.5 μg of FHV1(1,0)AKO or PaV1(1,0)AKO. Alternatively, cells were transfected with 2.5 μg of FHV1(1,0) or 5 μg of PaV1(1,0). FHV2(0,0) was transfected at 1 μg/well in the experiment shown in Fig. 5 and at 2 μg/well for that shown in Fig. 6.

Metabolic labeling of RNA

RNA replication products were labeled at 48 h posttransfection for 4 h by metabolic incorporation of [^{3}H]uridine in the presence of actinomycin D to inhibit DNA-dependent RNA synthesis (Ball, 1992). Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen) and each RNA sample (6 μg) was resolved in a 1.5% agarose-formaldehyde gel (Lehrach et al., 1977). Labeled RNAs were visualized by fluorography (Laskey and Mills, 1975).

Northern blot analysis

Total cellular RNA was extracted at 54 h posttransfection and incubated for 30 min at room temperature with 30 Kunitz units of RNase-free DNase I (Qiagen). Three micrograms of each RNA sample were resolved in denaturing agarose-formaldehyde gels as above and transferred to nylon membranes (Nytran SuPerCharge, Schleicher and Schuell). Hybridization with RNA probes was as described by Price et al. (2000). Positive-sense FHV RNA3 and RNA1 were detected using ^{32}P-labeled RNA probes that were complementary either to RNA1 nt 3008–3107 (Figs. 1B and 4) or to nt 2749–3107 (Fig. 6). Positive-sense FHV RNA2 was detected using either an RNA probe complementary to nt 773–1400 (Fig. 6) or an oligodeoxynucleotide complementary to nt 230–252 (Fig. 7). Positive-sense PaV RNA1 and RNA3 were detected using an RNA probe complementary to full-length RNA3 (Fig. 3). RNA probes were applied at between 2 and 4×10^6 cpm per membrane.

To achieve maximum strand specificity even under conditions of extreme asymmetry of +/− RNA levels, (+)-sense RNAs in Figs. 5 and 7 were detected using oligodeoxynucleotides as described before (Eckerle and Ball, 2002) except that hybridization was done at 47 or 49°C, respectively. For these experiments, (+)-sense FHV RNA3 species were detected using ^{32}P- end-labeled oligodeoxynucleotides complementary to RNA3 nt 345–363 (Fig. 5) or nt 48–69 (Fig. 7). Oligodeoxynucleotides were applied at between 2 and 5×10^6 cpm per membrane. Blots were exposed to a storage phosphor screen and visualized using a STORM-860 PhosphorImager digital radioactivity imaging system and ImageQuant v1.2 software (Molecular Dynamics).

Primer extension analysis of RNA

Total cellular RNA was extracted at 54 h posttransfection and DNase-treated as above. To map the 5′ ends of FHV RNA3 species, a ^{32}P-end-labeled oligodeoxynucleotide complementary to RNA3 nt 81–99 was annealed to 1 μg of each RNA sample and extended using 200 U of SuperScript III RNase H$^-$ reverse transcriptase (Invitrogen Life Technologies) at 55°C for 45 min. One-eighth of each primer extension reaction was resolved by electrophoresis in a 6% polyacrylamide sequencing gel alongside a dideoxynucleotide sequencing ladder generated by T7 Sequenase version 2.0 (Amersham Life Science) using the same end-labeled primer and plasmid FHV1(1,0) as template. Primer extension products and the sequencing ladder were visualized as for the Northerns above.

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